REMARKS

Applicant gratefully acknowledges the courtesy shown by Examiner Hunt at the personal interview conducted September 4, 2001. The constructive discussion during the interview clarified patentable subject matter in this application.

Claims 1, 4 and 18-24 have been canceled, without prejudice or disclaimer. Claims 2-3, 5-7, 9, and 12-17 have been amended. New claims 25 and 26 have been added. Accordingly, claims 2-3, 5-10, 12-17, and 25-26 are pending and are at issue.

Claim 5 has been amended to independent form, incorporating subject matter from canceled claims 1 and 4. This amendment is supported by the specification as filed, see *e.g.*, Example 15, page 52, line 30, to page 53, line 13 (schedule D).

Claims 2-3, 6-7, 9, and 12-17 have been amended to depend from claim 5 instead of canceled claims.

Claims 13-15 have also been amended to conform the claims to the language used in claim 5, *i.e.*, to clarify that the mammal or patient is a mammalian patient.

New claim 25 is supported throughout the specification, *e.g.*, at page 17, lines 3-6. New claim 26 is supported, *e.g.*, at page 15, lines 31-32.

No new matter has been added by way of this amendment.

NON-OBVIOUSNESS OF THE CLAIMED INVENTION

The Examiner has maintained the rejection of all claims under the judicially created doctrine of Obviousness-Type Double Patenting over claims 1 and 2 of U.S. Patent No. 5,290,551 to Berd (hereinafter Berd '551), in view of U.S. Patent No. 5,478,556 to Elliot (hereinafter "Elliot"), or Mankiewicz et al. (Cancer Immunol Immunother 1977;2:27-39; hereinafter "Mankiewicz"), or Humphrey et al. (Surg Oncol Gynecol Obstr 1971;March: 437-442; hereinafter "Humphrey").

The Examiner has also maintained the rejection of all claims under 35 U.S.C. §103(a) over Berd '551, or Berd et al. (Cancer Res 1991;51:2831-2734; hereinafter "Berd 1991"), or Berd et al. (Proc Am Assoc Cancer Res 1994;35:2731-2734; hereinafter "Berd 1994"), in view of Elliot, or Mankiewicz, or Humphrey.

While the Examiner concedes that the Berd references (Berd '551, Berd 1994, and Berd 1991) fail to teach weekly injections of vaccine, or administration of CY only prior to the first vaccine injection, the Examiner alleges that it would have been *prima facie* obvious to administer the composition weekly and administer CY prior to the first injection, as allegedly taught by Elliot, Mankiewicz, and Humphrey.

Because the basis for the rejections turns on the same references, applicants have considered them together, as in the prior response. The Examiner has also replied to applicant's prior response in a single argument.

As amended, the claims call for weekly administrations of vaccine where CY is only administered prior to the first vaccine dose. As described in the specification at page 17, lines 3-6 and in Example 15 (pp. 52-53), and discussed in the

previous amendment (mailed June 15, 2001), Applicant unexpectedly discovered that such a protocol had an advantage over protocols where CY was administered both before and after the first vaccine administration. It was also discovered that weekly vaccine administrations preceded by a single cyclophosphamide dose were more effective than administration every 28 days.

The omission of CY administrations after the first one, and the unexpected results that followed, are described in the specification and explained in the Declaration by the inventor, David Berd, accompanying the previous amendment. Briefly, the clinical protocol of monthly administration of melanoma vaccine was initially selected to permit injecting CY monthly, since CY was generally administered before every vaccine dose to inhibit suppression immune responses before vaccination, as reported, *e.g.*, in Berd '551, Berd 1991, and Berd 1994. Example 15 reports various attempts to modify this protocol. From this work, as the Examiner recognized, omitting all CY injections after the first vaccine dose, and retaining only the CY administration before the first vaccine dose, not only preserves but unexpectedly enhances the immune response (as measured by DTH response) as compared to the other protocols. These unexpected results are described in Example 15 of the present specification, and form the basis for the present invention.

As noted by the Examiner, two of the treatment groups in Example 15 received weekly administrations of vaccine for twelve weeks with either alternating haptenized and non-haptenized tumor cells (Group B) or haptenized vaccine only combined with three CY injections "spaced apart" during the treatment period (Group

C). Those groups showed a lower DTH response than Group A (standard 28-day protocol with CY before each dose), which itself was lower than the group receiving six weekly administrations of haptenized vaccine with only one CY injection given before the first vaccine dose. The Examiner then stated as follows (Office Action, p. 12, 2nd ¶):

> The declaration and post filing evidence clearly states that it is the single administration of cyclophosphamide administration..., in combination with an induction dose of vaccine and then weekly administration which achieves unexpected results. Further, the evidence supplied by the post filing evidence indicates that the specific enhance[d] DTH response is only achieved when a specific priming dose of cyclophosphamide is administered at a specific time, in combination with a proper priming dose.

In fact, various factors have been discovered by Dr. Berd subsequent to the filing of this application, that can improve the results of haptenized tumor cell immunotherapy of cancer. One of these factors is an induction dose (see Berd Declaration, ¶ 5), which is the subject of another patent application.[1] In the experiments described in the instant application, the group receiving a single administration of cyclophosphamide showed a higher DTH response than the groups receiving multiple CY injections. This difference in administration schedules was correctly noted by the Examiner, who recognized its significance (Office action, p. 12, 1st ¶). As discussed in the Berd Declaration (¶ 4), it could not be expected that the difference between the schedules of Groups C and D, *i.e.*, the omission of CY after the

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[1] This discovery was made in a retrospective analysis of clinical data, and thus is a separate invention.

first vaccine dose, would lead to such significant differences in DTH response. These results further emphasize the important discovery described in the specification and set forth by the present claims.

This unexpected advantage could not be predicted from any combination of the cited references. None of the references cited in the Office Action teaches or suggests that an immunotherapy regimen comprising weekly administrations of vaccine where no CY is given after the first vaccine administration would be as effective as one where additional doses of CY were given, much less that it would be better. On the contrary, Berd '551, Berd 1994, and Berd 1991 report successful immunotherapy with CY being administered prior to every dose of vaccine. Since tolerization by inhibitory lymphocytes is so important in suppressing immune response to tumor cells (see Exhibit A; Berd et al., 1986), and CY administration inhibits the suppressor cells (Id.), the prior art provides no incentive to modify a tumor immunotherapy schedule that cells for multiple doses of CY. Mankiewicz and Humphrey do not teach anything about CY administration. Only Elliot mentions that patients are "usually" treated with an anti-cancer drug, exemplifying CY, two to three weeks before vaccine administration, *i.e.*, for its tumorstatic rather than immunosuppressive effect. Thus, Elliot fails to mention the significance of excluding any subsequent CY administrations after initiation of the vaccine injection phase because it is not relevant to that reference's teachings.

Accordingly, there is no objective basis to improve or even modify the teachings of any of the Berd references, which report monthly administration of a

haptenized tumor cell vaccine with an adjuvant, each vaccine dose preceded by CY injection, to a schedule comprising a single dose of CY followed by weekly vaccine administrations. That such a major change of parameters, especially the exclusion of repeated doses of a drug (CY) believed to improve immunotherapy efficacy, would yield an enhanced immune response is counter-intuitive and could not have been predicted from any combination of the cited references. Thus, the method of the invention defies any reasonable expectation of success in view of the Berd references combined with Elliot, Humphrey, or Mankiewicz. Nor was any such deviation in, or improvement of, the inventive immunotherapy protocol suggested or implied by the combined references, since the administration of CY prior to each vaccine dose, as disclosed by all of the Berd references precludes weekly administration of the vaccine, as explained in the previous amendment. Accordingly, the references are facially defective in establishing *prima facie* obviousness of the claimed invention.

In view of the foregoing remarks, and the expert statements of Dr. Berd in the Declaration accompanying the previous amendment, it is clear that the Examiner's basis for rejecting the claims as obvious, whether under 103(a) or for obviousness-type double patenting, fails. For the reason set forth above, one of ordinary skill in the art would lack any motivation and reasonable expectation of successfully improving the therapeutic immune response by modifying the Berd vaccine as proposed by the Examiner.

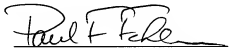
In view of the claims as amended herein and the foregoing remarks, applicant submit that the Examiner's rejections for obviousness are in error and should be withdrawn.

CONCLUSION

Applicants respectfully request entry of the foregoing amendments and remarks in the file history of this application. The claims are believed to be in condition for allowance. If the Examiner has any further questions, she is invited to contact the undersigned by telephone. Allowance of the claims is earnestly solicited.

Respectfully submitted,

Dated: September 27, 2001



Paul F. Fehlner, Ph.D.
Reg. No. 35,135
Attorney for Applicant(s)

DARBY & DARBY P.C.
805 Third Avenue
New York, New York 10022
212-527-7700

Exhibit A: Berd et al., Cancer Research 1986; 46:2572-2577

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David Berd

Serial No.: 09/304,859 Art Unit: 1642

Filed: May 4, 1999 Examiner: J. Hunt

For: COMPOSITION COMPRISING TUMOR CELL AND EXTRACTS AND METHOD OF USING THEREOF

MARK UP OF AMENDMENT
PURSUANT TO 37 C.F.R. § 1.121

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

2. (Amended) The method of claim [1]5, wherein said composition is administered for at least three times.

3. (Amended) The method of claim [1]5, wherein said composition is

administered for at least six times.

5. (Amended) [The method of claim 4]A method for inducing an anti-tumor response in a mammalian patient suffering from a tumor, which method comprises administering to said patient a composition comprising a tumor cell or tumor cell extract with an adjuvant, wherein the tumor cell or tumor cell extract is:

(i) conjugated to a hapten;

(ii) of the same tumor type as the patient's tumor;

(iii) not allogeneic to said patient, and

(iv) incapable of growing in the body of the patient after injection; and

repeating said administration at weekly intervals,

wherein a therapeutically effective amount of cyclophosphamice is administered only prior to the first administration of the composition, and wherein the composition, when administered with the adjuvant, elicits an anti-tumor response.

6. (Amended) The method of claim [4]5 wherein said therapeutically effective amount of cyclophosphamide comprises administering a dose of about 300 mg/M^2 of cyclophosphamide.

7. (Amended) The method of claim [1]5 wherein said tumor cell or

extract is selected from the group consisting of melanoma, lung, colon, breast, kidney, prostate, ovarian and leukemia tumor cell or extract.

9. (Amended) The method of claim [1]5 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, trinitrobenzenesulfonic acid, sulfanilic acid, arsanilic acid, dinitrobenzene-S-mustard and combinations thereof.

12. (Amended) The method of claim [1]5 wherein said adjuvant is selected from the group consisting of Bacillus Calmette-Guerin, QS-21, detoxified endotoxin and a cytokine.

13. (Amended) The method of claim [1]5 further comprising sensitizing [the] said mammalian patient with a therapeutically effective amount of the hapten prior to administering said composition.

14. (Amended) The method of claim [1]5 wherein said [mammal] mammalian patient is not sensitized to said hapten prior to administration of said composition.

15. (Amended) The method of claim [1]5 wherein said [mammal] mammalian patient is a human.

16. (Twice amended) The method of claim [1]5 wherein said composition comprises at least 10^6 tumor cells or cell equivalents extract per dose.

17. (Amended) The method of claim [1]5 wherein said anti-tumor response is at least one of the following: tumor necrosis, tumor regression, tumor inflammation, tumor infiltration by activated T lymphocytes, stable disease and prolongation of patient survival.

[CANCER RESEARCH 46, 2572–2577, May 1986]

Induction of Cell-mediated Immunity to Autologous Melanoma Cells and Regression of Metastases after Treatment with a Melanoma Cell Vaccine Preceded by Cyclophosphamide

David Berd,[1] Henry C. Maguire, Jr., and Michael J. Mastrangelo

Department of Medicine, Division of Medical Oncology, Thomas Jefferson University [D. B., H. C. M., M. J. M.], and the Department of Dermatology, Hahnemann Medical College [H. C. M.], Philadelphia, Pennsylvania 19107

ABSTRACT

There is considerable evidence in animal tumor systems that antitumor immunity is modulated by suppressor T-lymphocytes, and that the cytotoxic drug cyclophosphamide (CY) can abrogate that suppression. We measured the acquisition of delayed-type hypersensitivity (DTH) to autologous melanoma cells in 19 patients with metastatic malignant melanoma. The patients were treated with an autologous melanoma cell vaccine, either given alone, or given 3 days after the administration of CY, 300 mg/m² I.v. The DTH responses of CY-pretreated patients were significantly greater than those of control (vaccine only) patients. Thus, after two vaccine treatments, the median DTH responses (sum induration) were as follows: controls, 4 mm; CY pretreated, 11 mm; $P = 0.034$, Mann-Whitney U test, 2-tailed. Whereas seven of eight CY-pretreated patients developed DTH to autologous melanoma cells of at least 5 mm, only two of seven controls did so ($P = 0.034$, Fisher's exact test). Two patients had significant antitumor responses to treatment with CY plus vaccine, consisting of complete disappearance of skin metastases and a pulmonary nodule in one, and regression of s.c. and liver metastases in the other. Both patients remain free of melanoma after 42 and 33 mo, respectively.

INTRODUCTION

It is well established that immunotherapy can be effective against malignant tumors transplanted into experimental animals (1, 2). However, until recently, it was a commonly held belief that experimental immunotherapy could only be effective when administered before the inoculation of tumor cells, or shortly thereafter, *i.e.*, long before a tumor was palpable (3). The requirement for an extremely small tumor burden seemed to place a serious limitation on the usefulness of immunotherapy for human cancer (4).

It has now become clear that immunotherapy can cause regression of established, grossly evident murine tumors. For example, Berendt and North (5) have achieved immunologically mediated cures of a murine tumor as large as 5 mm in diameter. Such a tumor, having a mass of about 0.4 g, would be approximately equivalent to a 100-g tumor in an adult human patient (calculated on the basis of tumor mass/unit surface area).

This advance in immunotherapy has been made possible by a recognition of the fact that tumor-bearing animals have T-lymphocytes that can specifically suppress the immunological response to the tumor antigens (6–8). Several investigators have now shown that immunotherapy of established murine tumors can only be successful if steps have been taken to deplete or functionally impair these T-suppressor cells (5, 9). This can be accomplished by radical depletion (thymectomy plus whole-body irradiation) and then selective reconstitution of T-lymphocytes (5), or by administration of the cytotoxic drug, CY[2] (10–13).

We hypothesized that pretreatment with CY would enable patients with advanced cancer to develop cell-mediated immunity to tumor-associated antigens to which they would otherwise be unresponsive (12). Moreover, we reasoned that the development of immunity to those antigens could result in regression of metastatic tumor, providing the tumor burden was not too large.

We have tested these hypotheses in patients with metastatic malignant melanoma. We measured the acquisition of DTH to autologous melanoma cells after treatment with a whole cell vaccine, either given alone, or given 3 days after administration of CY. The results confirm that CY pretreatment markedly augments the development of DTH to melanoma-associated antigens, and that the resultant immunity can cause regression of metastatic tumors.

MATERIALS AND METHODS

Design of Study. The study population consisted of 19 patients with surgically incurable, metastatic melanoma. Eligible patients had to have one or more large (>2 cm) metastatic deposits that were easily resectable, *i.e.*, in s.c. tissue or superficial lymph nodes, and residual metastatic deposits that were measurable.

The patients were alternately assigned to one of two groups: (*a*) vaccine alone (control group) or (*b*) vaccine preceded by CY. The clinical characteristics of the patients in the two groups are shown in Tables 1 and 2. The distribution of age, sex, and Karnovksy status in the two groups was similar. Although all of the patients had s.c. and nodal metastases, four in the control group and six in the CY group also had visceral tumor deposits. All but two of the patients had been treated with cytotoxic chemotherapy. One patient in each group had received radiation therapy at least 6 mo prior to entry on this study.

The patients were tested and treated according to the scheme shown in Table 3. On Day 0, they were either left untreated or given CY, 300 mg/m², as an I.v. bolus. Three days later, all the patients were given injections of autologous melanoma vaccine. The treatment was repeated every 28 days. Prior to receiving vaccine, and 18 days after each vaccine injection, they were tested for DTH to autologous melanoma cells and to control materials (see below). Patients were continued on the study until there was clear evidence of progression of metastatic disease or until the supply of vaccine was exhausted.

Preparation of Tumor Cells. We used a modification of the method of Peters *et al.* (14). Freshly excised tumor masses were trimmed of skin, fat, and necrotic tissue and minced in cold modified Hanks' medium (Hanks' balanced salt solution plus 1% human AB-positive serum plus 0.1% EDTA plus penicillin plus streptomycin) (M. A. Bioproducts, Bethesda, MD). Cells that were released into the medium by mechanical dissociation were put aside and stored separately. The minced tumor pieces were placed in an enzyme solution, consisting of collagenase, 140 mg, and DNase, 30 mg, in 100 ml of modified Hanks' medium. The collagenase was type I (Sigma Chemical Co., St. Louis, MO) from *Clostridium histolyticum*; the DNase was type I (Sigma)

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Recipient of a grant from the National Cancer Institute (CA39248). To whom requests for reprints should be addressed, at the Division of Medical Oncology, Thomas Jefferson University, 1005 Curtis, 1015 Walnut St., Philadelphia, PA 19107.

[2] The abbreviations used are: CY, cyclophosphamide; DTH, delayed type hypersensitivity; DMSO, dimethyl sulfoxide; BCG, *Bacillus Calmette-Guérin*; I.d., intradermally.

Table 1 *Clinical characteristics of patients*

Patient	Sex	Age	Karnovksy performance status	Metastatic sites	Prior chemotherapy drugs	No. of vaccine treatments given	Survival times after starting vaccine (mo)
Cy pretreated							
1	M	58	90	S,[a] LU	DT	7	42+
2	F	61	70	N, LU	DT, meC	10	13
3	M	61	70	S	DT, V, CC, B, CY	2	6
4	F	46	90	S, LU	DT, CC	2	7
5	F	59	70	S, N	DT	3	5
6	F	61	70	S, LU	DT, CC, HN_2	2	6
7	M	48	50	N, LU	DT, CC, VBP	5	4
8	M	56	60	S, LI	DT, VI, CC, Vin	3	33+
9	M	31	90	S	None	12	32+
Controls							
10	M	59	90	S, N	meC, DT	3	11
11	F	76	90	LU, N	DT, CY	2	10
12	F	54	60	S, N	DT	2	3
13	M	59	90	LI, N	None	2	4
14	M	40	70	S, LI	DT, CC	2	7
15	F	61	60	S, BO, LU	CC, DT	2	6
16	F	48	90	S, N	V, P, DT	3	6
17	F	26	50	S, N	DT	2	6
18	F	52	50	S, N	meC, DT, T, CY, VBP	1	3
19	M	43	70	N	DT	2	1

[a] S, skin, LU, lung, DT, dacarbazine; N, nodal; meC, semustine; V, vinblastine; CC, lomustine; B, bleomycin; VBP, vinblastine-bleomycin-platinum; LI, liver; VI, vincristine; Vin, vindesine; BO, bone; P, platinum; T, Thiotepa.

Table 2 *Clinical characteristics of patients: summary*

	CY treated	Controls
No. of patients	9	10
Male/female	5/4	4/6
Age (median)	58 (31–61)[a]	54 (26–76)
Visceral metastases	6	4
No. of prior chemotherapies (median)	2 (0–5)	2 (0–7)
Karnovksy status (median)	70 (50–90)	70 (50–90)
Survival time (mo) (median)	7 (4–39+)	6 (1–11)

[a] Numbers in parentheses, range.

Table 3 *Outline of study*

Day of study	Procedure	
	CY group	Control group
−7	Apply skin tests	Apply skin tests
−5	Read skin tests	Read skin tests
0	CY, 300 mg/m² I.v.	No treatment
3	Vaccine 1	Vaccine 1
21	Apply skin tests	Apply skin tests
23	Read skin tests	Read skin tests
28	CY, 300 mg/m² I.v.	No treatment
31	Vaccine 2	Vaccine 2
49	Apply skin tests	Apply skin tests
51	Read skin tests	Read skin tests
56	CY, 300 mg/m² I.v.	No treatment
59	Vaccine 3	Vaccine 3

extracted from bovine pancreas. The dissociation process was carried out in baffled, trypsinizing flasks in a 37°C water bath with constant stirring using magnetic stir bars and an immersible stirrer. After 30 min of dissociation, the enzyme solution containing the cell suspension was removed, and fresh enzyme solution was added. The dissociation process was continued until no visible tumor tissue remained.

The tumor cells were washed twice in modified Hanks' medium, resuspended in freezing medium (RPMI 1640 plus 10% human AB-positive serum plus penicillin plus streptomycin plus 10% DMSO), and frozen in a controlled rate freezer (Union Carbide, Indianapolis, IN) at 1°C/min. They were stored in the liquid phase of liquid nitrogen until needed.

Preparation of Vaccine and Skin Test Material. On the day that a patient was to be skin tested or treated, the cells were thawed, and the DMSO was slowly diluted with modified Hank's medium. Then they were washed in Hanks' balanced salt solution without additives. The cells were irradiated in a cesium irradiator to a dose of 2500 R, washed, and resuspended in plain Hanks' at an appropriate concentration. The volumes were adjusted to a specific concentration of live tumor cells. Cells were identified as tumor cells by size and nuclear configuration. Contamination by dead cells (trypan blue exclusion), leukocytes, and erythrocytes varied from 10–30%. The number of live tumor cells injected did not vary appreciably between skin tests. Bacterial contamination was not observed either by direct microscopy or after short-term tissue culture experiments that were performed as part of an unrelated project.

Mechanically dissociated melanoma cells were obtained by collecting the material released by minced tumor pieces. Although the yields were low (14), sufficient numbers of viable cells were obtained for skin-testing 12 of the patients. Contamination of these preparations by dead cells and leukocytes far exceeded that of enzymatically dissociated cells and was sometimes as high as 90%.

The vaccine consisted of live tumor cells suspended in 0.2 ml of Hanks' solution to which was added BCG, 0.1 ml (approximately $0.8–2.6 \times 10^6$ viable organisms) (Glaxo Research Triangle Park, NC). The number of viable cells per vaccine treatment varied from $10–25 \times 10^6$, depending on the availability of material, but was similar in the CY and control groups [CY, 20 ± 2 (SE); control, 22 ± 1]. For seven of the patients (five CY pretreated, two vaccine only), two or more metastatic deposits were pooled to prepare vaccine. The tumor cell-BCG mixture was injected i.d. in three sites on the upper arms, alternating left and right, excluding arms ipsilateral to an axillary lymph node resection.

DTH Reactions. The patients were skin tested with each of the following materials suspended or diluted in 0.1 ml of Hanks' balanced salt solution: (*a*) 1×10^6 melanoma cells; (*b*) 3×10^6 autologous peripheral blood mononuclear cells, that had been separated on Ficoll-metrizoate and cryopreserved as described for the melanoma cells; and (*c*) a solution of collagenase and DNase mixed in the same proportions

as the solution used for dissociating tumor cells. The enzyme solution was used at the highest concentration (collagenase, 1.4 ng/ml; DNase, 0.3 ng/ml) that did not produce a primary irritant response in unimmunized subjects. DTH reactions were determined at 48 h by measuring the largest and right-angle diameter of the area of induration and calculating the mean. Biopsy of selected skin test sites showed the mononuclear cell infiltrate that characterizes DTH reactions.

RESULTS

DTH to Autologous Melanoma Cells. Prior to receiving vaccine, most patients did not exhibit significant DTH (*i.e.*, >5-mm induration) to autologous melanoma cells. The median responses were 3 mm in the control group and 2 mm in the CY group (not significantly different). Since it was impossible to determine whether these small skin test responses represented low-level cellular immunity or merely irritant responses, they were treated as "background" responses for statistical purposes. For all subsequent analyses, we subtracted the background response of each patient from his/her response measured after receiving vaccine.

The DTH responses to autologous melanoma cells after adminlstration of vaccine are shown in Fig. 1. The DTH responses of CY-pretreated patients were significantly greater than those of controls (vaccine alone); this difference was apparent when DTH responses were analyzed either after the first or the second vaccine treatment (CY group *versus* control group compared by 2-tailed Mann-Whitney *U* test: after one vaccine treatment, $P < 0.01$; after two vaccine treatments, $P = 0.034$). At the completion of two vaccine treatments, seven of eight CY patients, but only two of seven controls had DTH reaction greater than 5 mm ($P = 0.034$, Fisher's exact test).

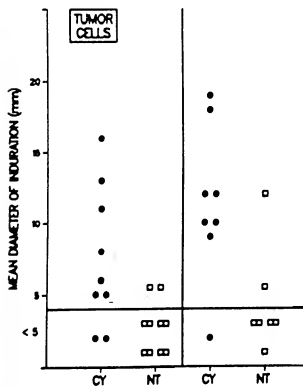

Fig. 1. DTH responses to autologous melanoma cells in patients treated with an autologous melanoma cell vaccine. The patients were either pretreated with CY or given vaccine with no pretreatment (*NT*). Each *point* represents the DTH response (mm, induration) of one patient. The differences between CY and no pretreatment were significant: after Vaccine 1, $P < 0.01$ after Vaccine 2, $P = 0.034$; 2-tailed Mann-Whitney *U* test.

In the CY group, DTH responses to autologous tumor cells were greater after two vaccine treatments than after one treatment. The mean increase in DTH between the first and second vaccine treatments was 4.0 ± 1.0 mm ($P < 0.02$, t test for nonindependent samples). Three CY-pretreated patients were skin tested at later time points. In two of these patients, DTH to autologous melanoma cells after three and six vaccine treatments was the same as the DTH response measured after two vaccine treatments. In the third patient DTH continued to increase after the fourth and sixth vaccine treatments.

Antitumor Responses—Vaccine Alone. None of the patients treated with vaccine alone showed any evidence of regression of metastatic tumor. All ten of these patients have died with a median survival time of 6 mo.

Antitumor Responses—CY Pretreated. Six of the CY-pretreated patients had progressive metastatic melanoma and have died with a median survival time of 6 mo. The other three patients are alive at 32+, 33+, and 42+ mo. Despite these long-term survivors, the difference in survival times between the CY and control group did not reach conventionally defined statistical significance ($P = 0.083$, Kruskal-Wallis analysis).

One CY-pretreated, long-term survivor (32+ mo) had very slowly progressive s.c. metastases while receiving immunotherapy (which was discontinued after 12 mo) but no evidence of tumor regression. The other two CY-pretreated survivors had significant antitumor responses with complete regression of all detectable metastatic melanoma. These two cases are presented in detail below.

Complete Responses to CY plus Vaccine. Patient 1 was a 58-yr-old man who presented with an advanced primary malignant melanoma on the anterior chest. There were dermal metastases of various sizes scattered over the anterior chest and both upper legs. Following chemotherapy with dacarbazine, the lesions on the skin became larger, and a single nodule (15-mm diameter) appeared in the right lung. At that time, treatment was initiated with CY plus vaccine, melanoma cells having been obtained by resection of several of the larger metastatic lesions in the skin. After two vaccine treatments, the lesions on the anterior chest were noted to be smaller. After seven vaccine treatments (approximately 7 mo), the skin metastases had regressed completely (Fig. 2), and the lung nodule had disappeared (Fig. 3). Treatment was discontinued at that time because the supply of vaccine had been exhausted. The patient remained free of disease until 6 mo later, when several small dermal metastases appeared on the right upper leg. These regressed completely after intralesional injection of BCG. The patient remains free of clinically detectable melanoma 42 mo after starting vaccine treatment.

Patient 8 was a 56-yr-old man who had a 2.8-mm-thick primary cutaneous melanoma removed from his left upper arm. Two yr later, he presented with multiple s.c. metastases over his trunk and abdomen, which failed to respond to chemotherapy with dacarbazine, vincristine, lomustine, and vindesine. At the time vaccine treatment was started, he not only had multiple (approximately 20) s.c. metastases, but he also had right upper-quadrant abdominal pain and an enlarged liver by physical examination. A liver scan showed several large defects characteristic of metastatic cancer (Fig. 4). The patient received three courses of CY plus vaccine over a 3-mo period, at which time the supply of vaccine had been exhausted. At the completion of

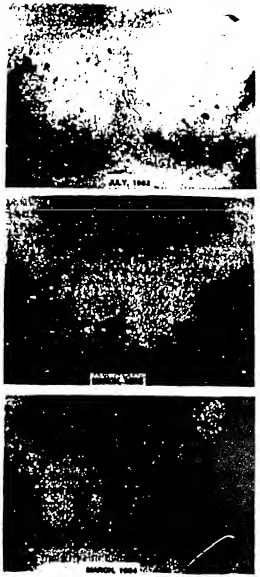

Fig. 2. Regression of skin metastases in Patient 1 after immunotherapy. The large residual pigmented lesion on the left breast is a benign (probably dysplastic) nevus.

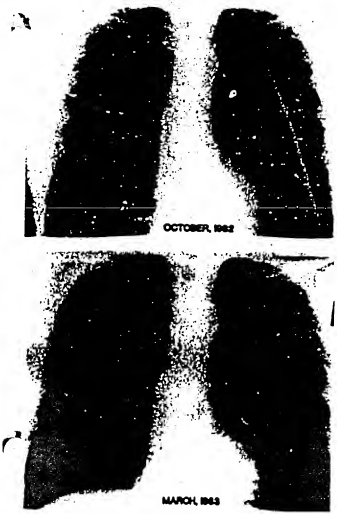

Fig. 3. Chest X-ray of Patient 1 before (*above*) and during (*below*) immunotherapy. Note right upper-lobe nodule (*arrow*) that disappeared during treatment with CY plus vaccine.

treatment, the s.c. metastases were noted to be smaller, and, 3 mo later, they had regressed completely. Another liver scan was performed which showed resolution of all the defects except one in the left lobe (Fig. 4); biopsy of that lesion and examination with melanin-specific stains showed melanin-containing cells with coagulation necrosis. The patient remained well until January 1984, 7 mo after beginning vaccine treatment, when he developed a solitary brain metastasis. This lesion was resected, and the patient was given a course of whole-brain irradiation. Twenty-six mo later (33 mo after starting immunotherapy), he is well and free of clinically evident melanoma.

Toxicity. The only toxicity noted was the local inflammatory response at the vaccine injection site. This consisted of a papule which became ulcerated and drained small amounts of clear fluid with healing by 3–4 wk after the injection. No patients developed fever, chills, or malaise. There were no clinical symptoms suggesting autoimmunity, such as arthralgias. Antinuclear antibody titers were in the normal range for all patients before and after vaccine treatment.

DTH Responses to Other Vaccine-related Antigens. No patients had DTH to autologous peripheral blood mononuclear cells, either before or after vaccine treatment.

Since the vaccine consisted of melanoma cells obtained by dissociation with collagenase and DNase, we tested DTH to those enzymes. No patient reacted to the enzymes before treatment, but after two courses of vaccine, 6 of 9 CY-pretreated patients and 4 of 7 controls had developed significant (>5 mm) DTH responses. For 12 of the patients (5, vaccine alone; 7, CY plus vaccine), we were able to obtain sufficient numbers of melanoma cells by mechanical dissociation, *i.e.*, not contaminated with the enzymes, in sufficient numbers for skin testing. Patients treated with vaccine alone did not develop DTH to autologous, enzyme-free melanoma cells, whereas 6 of 7 CY-pretreated patients did so. Thus CY-pretreatment augmented the development of DTH to melanoma-associated antigens and not just to the contaminating enzymes.

DISCUSSION

Several years ago, we provided the first evidence that CY can augment a human immune response (12). In patients with

Fig. 4. Regression of liver metastases in Patient 8 after immunotherapy. The pretreatment liver scan (*A*) shows multiple defects throughout the liver. Following CY plus vaccine, the scan (*B*) shows resolution of all the defects except one in the left lobe. Biopsy of that residual lesion was interpreted as necrotic melanoma cells.

advanced cancer, the development of DTH to the primary antigen, keyhole limpet hemocyanin, was markedly augmented by pretreatment with CY, 1000 mg/m² I.v. Subsequently we reported that a lower dose of CY, 300 mg/m², was equally effective in augmenting DTH and, in contrast to the higher dose, augmented the antibody response as well (13). The current study extends our previous observations by showing that CY immunopotentiation may also apply to tumor-associated antigens.

Pretreatment with CY markedly increased the acquisition of DTH to autologous tumor cells in this group of patients with metastatic malignant melanoma. Prior to vaccine administration, there were no significant DTH responses to autologous melanoma cells. This is consistent with previous reports (15) and confirms that evidence of tumor-directed, cell-mediated immunity is rare in patients with advanced cancer. Injection of vaccine alone was not effective in inducing DTH to autologous melanoma cells: only two of the control patients developed small positive reactions. In contrast, injection of vaccine with CY pretreatment 3 days before resulted in the development of DTH to autologous melanoma cells in all but one patient.

In two patients, the acquisition of DTH to autologous melanoma cells was followed by regression of metastatic tumor. This was almost certainly due to CY immunopotentiation: CY alone at this dose has no potential for causing tumor regression in malignant melanoma (13). Both responding patients exhibited complete regression of all cancer and are alive and well 42 and 33 mo, respectively, after beginning vaccine treatment. Following successful immunotherapy, patient 1 developed a new dermal metastasis that regressed after injection with BCG, and Patient 8 developed a solitary metastasis in an immunologically privileged site, the brain, that responded to surgery and irradiation. Despite the need for local therapeutic intervention, it seems likely that the current disease-free status of these patients is related to melanoma-directed immunity resulting from the CY plus vaccine administration. However, these therapeutic results must be considered preliminary until confirmed in a larger trial.

Both responding patients had relatively small cutaneous metastases which might have rendered them more "favorable" candidates for immunotherapy. However, there is strong evidence that visceral metastases also regressed. Patient 1 had a pulmonary nodule that appeared under observation and regressed after treatment. Patient 8 had symptoms and liver scan abnormalities characteristic of liver metastases which resolved after CY plus vaccine administration. A biopsy of a residual hepatic defect showed necrotic melanoma cells.

The failure of the other seven CY-pretreated patients to respond could be explained by the size of their tumor burdens. Although quantitation of human tumor burdens is imprecise, we estimate the tumor burden of Patient 1 at less than 100 g, that of Patient 8 at about 200 g, and those of other patients at 500 g or more.

The specificity of the DTH responses that we observed is an important issue, but one that is difficult to address with our current technological ability. The observation that our patients did not exhibit DTH to autologous blood mononuclear cells implies that neither components of the cell suspension medium (*e.g.*, antibiotics, allogeneic human serum, DMSO) nor cellular changes induced by the cryopreservation process were responsible for inducing DTH. However, it is clear that these patients developed immunity to the enzymes used for tumor dissociation as well as to melanoma-associated antigens. Ideally, one would like to immunize and skin test patients with a purified melanoma-specific antigen. Although such antigens have been isolated (16), they are not yet available in sufficient quantity for clinical use.

There is mounting evidence that active immunotherapy can produce clinically significant antitumor effects. McCune *et al.* (17) reported 4 tumor regressions in 14 patients with renal carcinoma treated with a vaccine made of enzymatically dissociated, intact autologous tumor cells. Tykkä *et al.* (18) have achieved regression of metastases in renal carcinoma patients using a polymerized protein extract as vaccine. Hoover *et al.* (19) have reported encouraging, although preliminary, results in a postsurgical adjuvant trial with colorectal carcinoma.

These reports coupled with our current findings suggest that active immunotherapy could become an important therapeutic modality in human cancer, if it is based on an understanding of the tumor-host relationship. Our demonstration that CY pretreatment augments the development of cell-mediated immunity to autologous melanoma cells suggests that tumor-directed T-suppressor cell activity is an important component of that relationship.

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